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Title: Mixed T-Cell Chimerism after Allogeneic Hematopoietic Stem Cell Transplantation for Severe Aplastic Anemia Using an Alemtuzumab-Containing Regimen is Shaped by Persistence of Recipient CD8 T Cells

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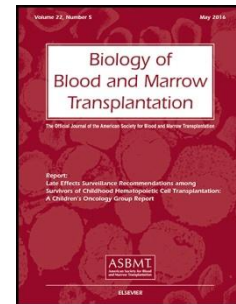
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Mixed T-cell chimerism after allogeneic hematopoietic stem cell transplantation for severe aplastic anemia using an alemtuzumab-containing regimen is shaped by persistence of recipient CD8 T cells

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Short title: Lymphocyte composition after HSCT using alemtuzumab for SAA

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Highlights

- Clinical outcomes are excellent using an alemtuzumab-containing HSCT regimen for aplastic anemia
- Outcomes are excellent despite prolonged abnormality of the T-cell profile
- Recipient-derived CD8 T cells shape persistent mixed chimerism

Abstract

Prevention of graft versus host disease (GVHD) is paramount for allogeneic hematopoietic stem cell transplantation (HSCT) to treat non-malignant diseases. We previously reported that allogeneic HSCT for severe aplastic anemia (SAA) using the fludarabine, cyclophosphamide and alemtuzumab ('Campath') (FCC) regimen is associated with very low risk of GVHD and excellent clinical outcomes. We now report a single center study of 45 patients with longer follow up and investigation of lymphocyte recovery. Overall survival (OS) was 93% and event-free survival (EFS) 90.7%. Acute and chronic GVHD occurred in 6 (13.3%) patients, respectively, and only one case was severe. Mixed T-cell chimerism was frequent and persisted after cessation of post graft immunosuppression. T cells were extensively depleted comprising only 11.3% of lymphocytes at day 30 rising to 43.8% by one year, but still significantly below normal (67.2%, $P=0.018$) and deficiency persisted after immunosuppressive therapy withdrawal. Depletion of CD4 T cells was particularly profound causing inversion of the normal CD4 to CD8 T-cell ratio. T-cell subset composition was also abnormal, with memory and effector T cells predominate for at least six months after FCC HSCT. Analysis of T-cell subset chimerism showed CD4 T cells were predominantly donor-derived at one year whereas recipient-derived CD8 T cells shaped mixed chimerism with notable contribution of recipient effector CD8 T cells. Prolonged mixed T-cell chimerism after withdrawal of post graft immunosuppression and low incidence of GVHD indicates mutual tolerance is

established, but incidence of viral disease was low suggesting that anti-viral immunity is maintained. The study showed that despite abnormality of the T-cell profile after allogeneic HSCT for SAA using the FCC regimen, it is conducive for excellent clinical outcome.

Introduction

In contrast to hematologic malignancies where GVHD has an important graft versus leukemic effect, there is no advantage for any GVHD in allogeneic HSCT for non-malignant diseases. *In vivo* T-cell depletion (TCD) is used to reduce GVHD. We pioneered the use of alemtuzumab instead of antithymocyte globulin (ATG) for allogeneic HSCT with TCD to treat SAA. Using the 'FCC' conditioning regimen of fludarabine, low dose cyclophosphamide with alemtuzumab ('Campath-1H'), we previously reported in a retrospective multi-center study an OS of 85-90% and a remarkably low incidence of chronic GVHD^{1 2}. Subsequent studies have confirmed the benefit of alemtuzumab over ATG in reducing both acute and chronic GVHD in SAA HSCT^{3 4 5}. A recent large multicenter study examining cyclophosphamide dose de-escalation in unrelated donor HSCT for SAA using ATG, confirmed excellent OS but the 1-year incidence of chronic GVHD was 22.5-31.7%, depending on cyclophosphamide dose used⁶. Allogeneic HSCT with alemtuzumab for TCD has also been applied to the treatment of sickle cell anemia and excellent clinical outcomes reported⁷.

Previous studies of chimerism post HSCT for SAA examining unfractionated blood mononuclear cells have demonstrated a significant incidence of mixed chimerism.

Although graft failure occurred in a high proportion of patient with progressive mixed chimerism, stable mixed chimerism was associated with a low risk of chronic GVHD⁸. Among FCC HSCT patients, we observed that stable mixed T-cell chimerism alongside full donor myeloid engraftment is frequent and persists after cessation of post graft immunosuppression¹. This has also been reported in a proportion of children transplanted for SAA using FCC regimen⁹. The very low incidence of GVHD and sustained mixed T-cell chimerism suggests that a state of mutual immunological tolerance exists after FCC HSCT. In this study, we have investigated the recovery of lymphocyte subsets in SAA patients transplanted at King's College Hospital using the FCC-conditioning regimen. We report the lymphocyte profile associated with excellent clinical outcomes and the basis for persistent co-existence of donor and recipient T cells.

Methods

Patients

From 2007 to 2015, 45 consecutive patients with acquired idiopathic SAA were transplanted at King's College Hospital. The diagnosis of AA¹⁰ and the disease severity^{11 12} was defined using standard criteria. Fanconi anemia was excluded by diepoxybutane stimulation of cultured peripheral blood chromosomes. Patients with likely constitutional AA, based on an identified mutation, family history, and/or somatic anomalies but no identified mutation, were excluded. Patient characteristics are summarized in Table 1. National Research Ethics Committee approval was obtained to collect patient samples for research studies via the King's College London Haemato-Oncology Tissue Bank, and informed written consent was obtained in

accordance with the Declaration of Helsinki.

Transplant conditioning regimen

High resolution DNA typing for HLA-A, B, C, DRB1 and DQB1 was performed for all patients. The FCC conditioning regimen was used for 10/10 HLA-matched HSCT patients, as previously described ¹. FCC conditioning with 2Gy total body irradiation was used for 9/10 HLA-matched HSCT patients. Median alemtuzumab dose was 70mg (range 45-100). Cyclosporine (CSA) alone was used as post HSCT IST. Four patients later required addition of mycophenolate mofetil due to renal impairment that precluded full dose CSA. Target trough CSA blood levels were 250-350 mcg/l. IST was continued for 9 months post HSCT, followed by tapering over the subsequent 3 months, provided hematologic parameters and mixed T-cell chimerism were stable.

Lymphocyte phenotyping

Twenty-nine patients underwent longitudinal multi-parameter flow cytometric analysis of lymphocyte subset reconstitution. Patients who had prednisolone or rituximab were excluded after treatment commenced. Analysis of 11 adult healthy volunteers was also performed. Cryopreserved peripheral blood mononuclear cells were thawed and labeled with fluorochrome conjugated antibodies to characterize lymphocyte subsets. Analysis was performed by flow cytometry using a BD FACSCanto II or a BD LSRFortessa. Results were analyzed with FlowJo software. Dead cells were excluded from the analysis based on their forward- and side-light scatter properties and use of a Zombie Fixable dead cell staining kit (Biolegend). Monocytes were excluded based on expression of CD14. NK cells were defined as

CD3-/CD19-/CD56+. B cells were defined as CD19+. CD4 T cells were defined as CD3+/CD4+ and CD8 T cells as (CD3+/CD8+) with naïve (CD45RA+/CD27+/CD62L+), memory (CD45RA-/CD27+), effector (CD45RA-/CD27-/CD62L-) and terminal effector (CD45RA+/CD27-/CD62L-) subsets.

Chimerism

Chimerism was assessed in unfractionated peripheral blood, CD3+ T cells and CD15+ granulocytes. Full donor chimerism was defined as > 95% donor hematopoietic cells. Lymphocyte subsets were isolated using a BD FACSAria II cell sorter (purity >95%). Genomic DNA was extracted using a QIAamp DNA Micro kit (Qiagen). Percentage donor chimerism was determined using the PowerPlex 16 HS (Promega) multiplex short tandem repeat system for DNA typing. Genetic analysis was performed using an Applied Biosystems 3130xl and results interpreted using ChimerMarker (Version 3.0.9).

Statistical analysis

Univariate comparisons and multivariate analysis was performed using the Cox proportional hazards regression model with Statistical Package for the Social Science (SPSS) software. Comparisons of lymphocyte subset composition in patients and healthy volunteers were made with the Mann-Whitney U test using GraphPad Inc. software, Prism version 6.0. All tests were 2-tailed, with *P*-values <0.05 considered to be statistically significant. All data were censored as of 30 December 2015.

Results

Clinical outcomes after FCC HSCT

In this single center study, we have confirmed the excellent clinical outcomes as previously reported following HSCT using FCC conditioning for SAA¹ (Table 2). The median patient follow-up after HSCT was 31.4 months (range 3-93) with excellent OS and event-free survival (EFS) at five years of 93 and 91% respectively. Importantly there was no significant difference in outcomes for MSD compared to UD transplants or for patients aged > 50 years (n=14), compared to younger patients. Three patients died, resulting in a TRM rate of 7% at one year. The rate of GVHD was very low; the majority of which was mild. Reliable and sustained engraftment was observed with only one graft failure noted in a patient who received a suboptimal bone marrow infusion cell dose. Sequential chimerism data were available for 42 (93%) patients that confirmed the persistent mixed T-cell chimerism previously reported with the FCC conditioning regimen¹ (Figure 1A).

Epstein-Barr virus (EBV) viremia was detected in 20 (47%) patients, but treatment was only required in two (5%) patients. One patient developed biopsy-proven EBV post-transplant lymphoproliferative disease (PTLD) on day 120 and the second had excessive EBV viral load on day 66 but no signs of PTLD; Cytomegalovirus (CMV) viremia was observed in 11 (42%) patients, but no patients progressed to CMV disease due to pre-emptive therapy. Adenovirus viremia was observed in 3 (7%) patients. Invasive fungal infections were diagnosed in three (7%) patients, two with a fatal outcome who both had the infection pre-transplant.

Post FCC HSCT, 9 patients (20%) developed autoimmune-like pathologies; 4 with hemolytic anemia, 4 with pure red cell aplasia, (1 of whom also had immune

thrombocytopenia and 1 who had thyroiditis) and 1 with probable immune-mediated neutropenia that responded to granulocyte colony stimulating factor (supplementary Table 1. All 4 cases of pathology classified as warm type autoimmune hemolytic anemia (AIHA) responded to prednisolone but 3 relapsed, 2 of 2 responded to rituximab. The other patient, who received a 9/10 HLA-match HSCT, had refractory and fulminant hemolysis in association with severe GVHD and multiple thromboses. Of the 4 cases of pure red cell aplasia (PRCA), 3 received a major ABO mis-matched transplant that is the likely cause of their PRCA. Three of four patients recovered attaining transfusion independence while in one patient the PRCA was refractory to steroids, intravenous immunoglobulin, rituximab, and donor lymphocyte infusion. This patient has received a second HSCT from an alternative ABO matched MUD.

Lymphocyte reconstitution after FCC HSCT

Serial analysis of peripheral blood lymphocytes of 29 patients after FCC HSCT showed the expected lymphopenia associated with use of alemtuzumab, and total number of lymphocytes remained significantly below normal ($P<0.0001$) at one year and beyond (Figure 1B). NK cells were the predominant lymphocyte population present early post HSCT comprising 49.3% of lymphocytes at day 30 (Figure 1C). B cells were not detected at day 30, but rapidly recovered comprising 31.7 of lymphocytes at day 60 (Figure 1C) and frequency remained significantly above normal at day 360 (23.7% compared to 10.5% for healthy volunteers, $P=0.002\%$).

T-cell deficiency is prolonged and the CD8 T-cell population is skewed to an effector phenotype

T cells were profoundly deficient, comprising only 11.3% of lymphocytes at day 30,

increasing to 43.8% at one year, but still significantly below normal (67.2%, $P=0.018$) (Figure 1C). Rapid recovery of B cells with prolonged T-cell deficiency produced abnormal dominance of B cells over T cells, maintained long term. Frequencies of CD4 T cells within the T-cell population were lower than normal producing inversion of the usual CD4 to CD8 T-cell ratio (Figure 1D). Memory and effector T cells predominated early post HSCT (Figure 1E). Naïve T cells began to recover from 6 months (Figure 1E). Composition of naïve, memory and effector subsets within the CD4 T-cell population were near normal at one year (Figure 1E left panel). In contrast, CD8 T-cell subset composition remained abnormal at one year due to a high proportion of effector T cells (57.2% compared to 9.3% for healthy volunteers, $P<0.0001$) (Figure 1E right panel).

Persistence of recipient CD8 T cells after FCC HSCT

Lymphocyte subsets from five patients that were mixed T-cell chimeric at one year were isolated by fluorescence-activated cell sorting, and donor / recipient composition determined by analysis of informative alleles from polymorphic short tandem repeat loci. Four patients were >93% donor NK cells and >76% donor B cells. Patient 3 was only 39% donor NK cells and 76% donor B cells. Naïve, memory and effector T-cell subsets were isolated using the gating strategy shown Figure 2A. Results shown in Figure 2B revealed that mixed T-cell chimerism at one year was principally due to persistence of recipient CD8 T cells. Median percentage contribution to recipient CD3 chimerism was 37.9% for the CD8 T-cell population and 8.5% for the CD4 T-cell population ($P=0.008$). Recipient chimerism was detected in all CD8 T-cell subsets, but with notable contribution of the effector subset in patients 3 and 4 who both had CMV reactivation early after HSCT. A significant correlation was observed between

lower donor T-cell chimerism at day 360 and CMV reactivation or EBV viraemia early after HSCT. Patients that were donor and/or recipient CMV/EBV seropositive but had no viraemia post HSCT were median 77% donor CD3 compared to median 57% donor CD3 for patients that had viraemia post HSCT ($P=0.036$). Follow-up analysis of patients 1, 2 and 3 at >2 years post HSCT showed that the T-cell subset mixed chimerism profile remained stable after IST cessation and numbers of CD4 and CD8 T cells did not increase (data not shown).

Discussion

Using the FCC conditioning regimen for allogeneic HSCT to treat SAA at a single center and with long-term follow up, we confirm the excellent clinical outcomes previously reported in our multi-center study ⁵. Incidence of chronic GVHD was remarkably low, particularly given the high proportion of MUD in this cohort. The comparable outcome of older (>50 years of age) and younger patients is also encouraging, given that increasing age is typically associated with worse outcome ¹³. Rapid full donor myeloid engraftment occurred and there was only one case of graft failure, associated with low infused bone marrow stem cell dose. The majority of patients exhibited sustained mixed T-cell chimerism that persisted despite withdrawal of immunosuppression at one year after transplantation.

The alemtuzumab-containing FCC regimen caused profound and prolonged T-cell depletion, particularly CD4 T cells that recovered more slowly than CD8 T cells. The small numbers of T cells that survived depletion with alemtuzumab were almost exclusively of the memory or effector subsets and dominated the circulating

population for at least six months. We previously reported the same selective preservation of antigen-experienced T cells after HSCT using a fludarabine, busulphan with alemtuzumab regimen for treatment of patients with hematological malignancies¹⁴. The very slow recovery of T cells is likely due to thymic atrophy in adults limiting *de novo* production of naïve T cells after HSCT. The combination of T-cell depletion and the relatively mild conditioning with fludarabine and low dose cyclophosphamide that is unlikely to induce a strong cytokine storm limits potential for T-cell alloreactivity. Of the seven patients who developed GVHD, six were >90% donor CD3 and one was 75% donor CD3 by day 60.

Mixed T-cell chimerism is frequent after reduced intensity conditioning and particularly common after TCD with alemtuzumab¹⁵. Our investigation of mixed chimerism within T-cell subsets found that CD4 T cells were mainly donor-derived by one year but CD8 T cells remained predominantly recipient-derived. Although naïve, memory and effector subset frequencies in the CD4 T-cell population were near normal by one year, numbers were low in all patients and deficiency persisted after cessation of IST. In contrast, recovery of CD8 T cells was much more robust, but an atypical composition developed due to expansion of the effector CD8 T-cell subset that rose from a mean of 17.6% CD8 T cells at day 90 to 57.2% at one year (compared to 9.3% for healthy volunteers). Recipient cells were present in all CD8 T-cell subsets, but was notably high in the effector population and particularly for the two patients who had CMV reactivation early after HSCT.

Expansion of the effector CD8 T-cell population occurred despite continued IST for at least one year. CMV reactivation and EBV viremia early after transplantation

correlated with higher recipient CD3 chimerism at one year and we therefore speculate that expansion of effector CD8 T cell numbers may be due to antigen-driven proliferation of pathogen-specific recipient CD8 T cells. Limited sample availability and low T-cell numbers precluded our investigation of the antigen-specificity of the effector CD8 T cells. The incidence of viral disease post FCC was low, despite EBV and CMV viremia in 47% and 42% of patients respectively. Recipient-derived effector CD8 T cells may confer a degree of anti-virus immunity after FCC HSCT, despite continued IST. Other studies have recently reported that virus-specific CD8 T cells can have a substantial impact on T-cell composition after HSCT. Massive expansion of CMV-specific effector CD8 T cells is seen following CMV reactivation after HSCT and has a long-term impact on T-cell reconstitution^{16 17}. In the setting of TCD using alemtuzumab-based reduced intensity conditioning in patients transplanted for hematologic malignancies, Sellar and colleagues showed that CMV-specific CD8 T cells were of recipient origin and contributed significantly to higher recipient chimerism¹⁸.

For patients with hematological malignancies, routine clinical practice is to administer donor lymphocyte infusions (DLI) for mixed T-cell chimerism to promote conversion to full donor chimerism and prevent disease relapse¹⁹. DLI is not administered to SAA patients after FCC HSCT because disease relapse does not occur and DLI has the associated risk of GVHD. Our study of lymphocyte composition and chimerism has provided novel insight into the long-term impact of HSCT conditioning with alemtuzumab. Stable co-existence of donor and recipient T cells for many years after withdrawal of immunosuppression, reliable engraftment and low rates of GVHD indicates that a state of mutual tolerance exists after allogeneic FCC HSCT. HLA-

matching, T-cell depletion using alemtuzumab together with IST for at least one year after HSCT is an effective strategy to minimize alloreactivity. Although our study showed the FCC HSCT regimen results in an atypical lymphocyte composition, clinical course and outcomes for patients are remarkably good.

The only complication consistently observed after FCC HSCT is emergence of autoimmune-like diseases that are predominantly antibody-mediated cytopenias, seen in 6 (14%) patients in this study. Secondary autoimmune disorders commonly occur after treatment with alemtuzumab in other clinical settings²⁰. Prolonged T-cell deficiency may contribute to dysregulated B-cell behavior. We have previously reported that clonal gammopathies are frequent after using alemtuzumab-based HSCT²¹. No association of autoimmune-like disease with circulating B cells or other lymphocyte populations was found in our study (data not shown).

In conclusion, despite persistent T-cell deficiency, excellent survival and low incidence of viral disease was observed after FCC HSCT for SAA. The very low incidence of GVHD with long-term stable mixed T-cell chimerism, shaped by recipient CD8 T cells, in the absence of immunosuppression indicates that FCC conditioning favors mutual tolerance.

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Figure Legends

Figure 1. Serial analysis of peripheral blood chimerism and lymphocyte composition after FCC HSCT. (A) Percentage donor chimerism of unfractionated, purified CD3 and purified CD15 peripheral blood cells. Mean and SEM are shown. (B) Reconstitution of peripheral blood lymphocytes after FCC HSCT. Median and interquartile range are shown and the horizontal dotted lines enclosing grey boxes represent the median and interquartile range of 11 adult healthy volunteers. Comparisons between cell numbers at each time point and healthy volunteers were

performed using a 2-tailed Mann-Whitney *U* test. Lymphocyte numbers at all time points were significantly below numbers for healthy volunteers ($P < 0.0005$). (C) Percentage of NK, B and T cells. Mean and SEM are shown and values for 11 adult healthy volunteers (HV) indicated. (D) Percentage of CD4 and CD8 T cells within the CD3 T-cell population using mean values. (E) Percentage naïve, memory and effector subset composition of CD4 T cells (left panel) and CD8 T cells (right panel). Mean and SEM are shown and values for 11 adult healthy volunteers (HV) indicated.

Figure 2. Analysis of individual patients showed sustained mixed T-cell chimerism at after FCC HSCT is primarily due to persistence of recipient CD8 T cells, with notable contribution of the effector subset. (A) Panels illustrate the gating strategy used for isolation of naïve, memory and effector T-cell subsets by FACS. (B) CD4 T-cell (left panel) and CD8 T-cell (right panel) subset chimerism at one year for five patients. CMV serostatus donor/recipient and reactivation early post HSCT: Patient 1 negative/negative, Patient 2 negative/positive no reactivation, Patient 3 positive/negative and reactivation, Patient 4 negative/positive and reactivation, Patient 5 negative/positive no reactivation.

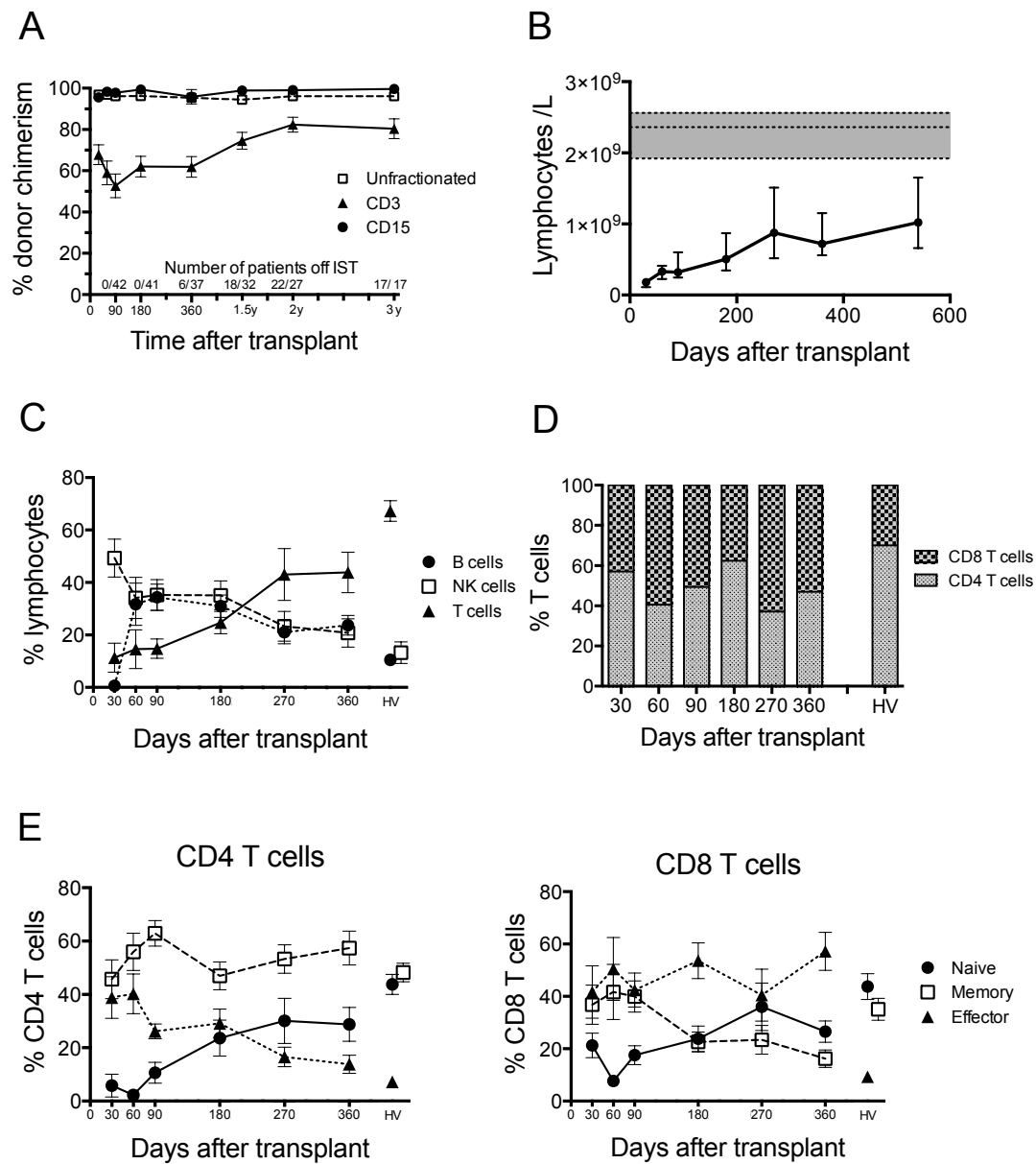
Figure 1

Table 1: Patient pre-transplant characteristics

Number of patients	45
Median age (range) Number of patients aged > 50yr	32 (15 – 63) 14 (31.1%)
Male / Female	28 / 17
Etiology of aplasia <ul style="list-style-type: none"> Idiopathic Post-hepatic (seronegative) Eosinophilic fasciitis Coeliac disease 	41 (91.2%) 2 (4.4%) 1 (2.2%) 1 (2.2%)
Type of donor <ul style="list-style-type: none"> Matched sibling (MSD) Unrelated donor (UD) 9/10 UD 	12 (26.6%) 33 (73.4%) 8 of 33 (24.2% of UD)
Previous immunosuppressive therapy <ul style="list-style-type: none"> MSD UD 	6 of 12 (50%) 27 of 33 (81.8%) $p = 0.055$
Median time to transplant <ul style="list-style-type: none"> MSD UD 	5.9 (2.7-34.7) 8.4 (2.1-178.9) $p = 0.095$
Number of patients HLA alloimmunised	11 (24.4%)
Stem cell source <ul style="list-style-type: none"> Bone marrow Peripheral blood stem cells 	7 (15.5%) 38 (84.5%)
Number of patients with PNH clone Median PNH clone size: <ul style="list-style-type: none"> Granulocytes Monocytes Red cells 	21 (46.6%) 2% (range 0.02 – 40) 2.85% (range 0.01 – 32) Not Available in the table
Median alemtuzumab dose	70 mg (Range 45 – 100)
Median CD34+ stem cell dose	6.55×10^6 CD34+/Kg (Range 1.97 – 12.40)
Median follow up after HSCT	31.4 months (Range 3 – 93)

Table 2. Patient outcomes after FCC HSCT

Median time to neutrophil count $> 0.5 \times 10^9/l$	12 days (Range 10 – 22)
Median time to platelets $> 20 \times 10^9/l$	12 days (Range 9 – 61)
Primary graft failure	1 (2.2%)
Acute GVHD: ▪ Grade I/II ▪ Grade III/IV	6 (13.3%) ▪ 6 of 6 (100%) ▪ 0 of 6
Chronic GVHD: ▪ Mild ▪ Moderate ▪ Severe	6 (13.3%) ▪ 4 of 6 (66%) ▪ 1 of 6 (17%) ▪ 1 of 6 (17%)
1-year TRM	3 (6.6%)
5-year OS	93.1%
5-year EFS	90.7%
5-year EFS MSD (n=12) vs UD (n=33)	100% vs 87.4% ($P=0.219$)
5-year EFS Age ≤ 50 years (n=31) vs >50 years (n=14)	93% vs 85.7% ($P=0.356$)